Modeling human neuronal migration deficits in 3D

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Abstract

During the last few decades we have witnessed an impressive gain in the knowledge regarding the basic mechanisms underlying human neuronal migration disorders by the usage of mouse models. Nevertheless, despite the remarkable conservation both in the genetic encoded information and the developmental processes, there are still numerous important differences between human and mouse. This may explain the vast excitement following the realization that technological breakthroughs enabled generating tissue-like human-based organoids for modeling human neuronal migration diseases. This review will provide a short introduction on human and mouse neuronal migration processes, and highlight human brain organoid models of neuronal migration diseases.

Highlights

* Cajal-Retzius (CR) cell migration in human and mouse is similar yet different.

* The expanded outer-subventricular zone is human specific.

* Cortical organoids for modelling lissencephaly and periventricular brain heterotopia.

* Fusion organoids derived from patients demonstrated interneuron migration deficits.

1. Neuronal Migration Processes- Human vs. Rodents

Neuronal migration is a remarkable process in which newborn neurons in the developing brain move to their final destination. There they will make proper short distance and long-distance contacts and will form functional neuronal circuits. The human brain is far larger than that of the mouse, way more complex, and develops over a longer time scale. Whereas in humans, developmental neuronal migration occurs during months of gestation, and continues in the early postnatal period, in rodents this process is completed within days.

The first migrating neurons in the human and rodent developing brains are the transient Cajal-Retzius (CR) neurons that reside in the superficial layer (layer I) and the subplate and are eliminated in postnatal mice by a Bax-dependent apoptosis [1, 2]. In both of these locations the CR neurons have been shown to affect the development and proper organization of other neurons. These cells are born in the ventral pallium, the septum and the cortical hem [3], and adopt wide-spread tangential migratory routes (Figure 1A). CR cells are best known for their role in the neocortex where they reside in the marginal zone and secrete reelin, that is involved in directing projection neurons during their radial migration [4].
The identification of lack of Reelin as the naturally occurring reeler mutation encountered a well understood excitement in the scientific community [5, 6]. Reeler mice exhibit inverted cortical lamination and display staggering gait, poor balance, tremors and ataxia. Reelin regulates radial migration by providing both attractive signals to pre-migratory neurons [7] and detachment/stop signals at later stages of migration. Reelin function is mediated by the downstream signaling following its binding to either of its receptors VLDLR or ApoER2. Following ectopic reelin expression, binding to ApoER2 but not to VLDLR will cause aggregation, while VLDLR is required for suppressing neuronal invasion to the marginal zone [8]. Reelin is also important for human brain development and cortical lamination. Autosomal recessive mutations result in lissencephaly with cerebellar hypoplasia [9]. Beyond migration,

reelin shapes the synaptic architecture as its gradient attracts retinal axons arborization in the visual cortex [10]. Reelin continues to be expressed in sub population of GABAergic neurons in the adult brain where it appears to play a role in neuronal maturation, synaptic formation and plasticity. Indeed, conditional KO of reelin in the postnatal brain, caused hyperexcitability of the neocortical network [11]. These functions connect reelin to a much broader spectrum of neurological disorders than initially estimated, including not only lissencephaly, hypoplasia and epilepsy but also schizophrenia, autism, and neurodegenerative diseases such as Alzheimer's disease [6].

In the mouse brain (E10.5-12.5), CR cells migrate within the preplate to populate the caudo-medial, rostro-medial, and lateral cortex [12]. The CR neurons respond to external cues controlling their migration. For example, hem-derived CR neurons respond to CXCL12/CXCR4 signaling originating from the meninges [13]. Genetic ablation of septum Dbx1-derived CR cells affected the migration of CR cells from other origins and this perturbation resulted in changes in the early regionalization of the neuroepithelium [12]. Recent studies demonstrated that in the mouse brain, a secreted form of the transcription factor PAX6 is involved in regulation of CR cells derived from the cortical hem and septum in a non-cell autonomous way [14]. The CR neurons that reside in the subplate play an important role in establishing the corticothalamic innervations [15]. In humans, the CR neurons are more diverse and contain transient and persistent populations [16]. The human-mouse difference may originate in part from differences in the regulation of expression of important transcription factors, such as DBX1 [17]. The expression of Dbx1 in the mouse is confined to cortical progenitors in the ventral pallium, however in primates it is maintained in neurons. Modifying the expression pattern of Dbx1 in mouse to resemble the primate expression resulted in ectopic CR cells and subplate neurons. Similar to the mouse, the first human CR neurons appear shortly after the formation of the telencephalic vesicle at Carnegie

stage (CS) 16 (5 GW) [18]. A human specific organization is observed at CS 17-19, when CR cells are born in columns in the neuroepithelium of the anterior cortex and spread into the marginal zone. In addition, they are born in the subpallium and the septum (Figure 1A). At the onset of gyration, most transient CR neurons die in an activity-dependent programmed cell death that is critical for correct wiring of functional cortical circuits and the maintenance of proper excitation/inhibition balance [2]. These CR neurons are replaced by the persisting ones [16]. A recent study suggests that in humans subplate neurons migrate and differentiate into deep layer projection neurons [19].

The majority of the neurons in the cerebral cortex are the excitatory or the glutaminergic neurons, that are born either within the ventricular zone or in the subventricular zone [20-24] (Figure 1B). In the rodent brain, the progenitors of these neurons are the apical radial glia (RG) progenitors that proliferate close to the ventricle and are integrated into the apical adherens junction belt during cell division, and the basal progenitors (BP) that lack the typical polar morphology of the apical progenitors and undergo mitosis in a basal position [20]. The BPs pool is composed by intermediate progenitor cells (IPCs) and basal radial glial cells (bRG). The later, possess a basal process and are relatively nonabundant in the rodent developing cortex [20].

Initially, there is an accelerated proliferative division of neural stem cells, followed by the emergence of RG [24]. Many of these progenitors span the entire cortical wall, while others have short processes known as short neural or subapical precursors [23, 25]. The RG undergoing symmetric proliferative divisions constantly regenerate the apical endfeet. The shift to the neurogenic stage is regulated by signaling pathways that include Notch and ß-integrin and is accompanied by a decline in the ability to generate the apical prosses and basal translocation that promotes the formation of the outer subventricular zone (oSVZ) [26]. The

RG display typical interkinetic nuclear movements, where the position of the nucleus is coupled to the cell cycle, giving the ventricular zone its packed pseudostratified appearance [21]. The formation of layers of neurons in the emerging cortex depends on the consecutive formation of waves of radially migrating cells in an "inside - out" fashion, the earlier born neurons are located in deep layers and the later born neurons are located in more superficial layers [27]. This process is conserved in human and mouse (Figure 1B). Newly generated neurons display multipolar morphology, and migrate slowly in the subventricular and intermediate zone [28]. The transformation of the multipolar cells into bipolar neurons is regulated by subplate neurons [29]. The subplate neurons signal to the following waves of radially migrating neurons by forming transient glutamatergic synapses. The synaptic transmission induces the typical multipolar-to-bipolar morphology, that marks the transition from slow multipolar migration to faster pia-oriented RG-guided migration [29].

The human developing brain is distinguished by a huge expansion of the subventricular zone, generating a new region called the oSVZ [20, 22]. In this area, outer radial glia (oRG) can be found. The expansion of this area is due to multiple cell divisions of these progenitors during which they exhibit mitotic somal translocation, a typical behavior in which the cell soma rapidly translocates towards the cortical plate prior to cytokinesis [20, 22, 24]. It has been suggested that during cortical expansion, the processes of the apical RG do not extent to the whole width of the cortex, therefore the migrating neurons possibly migrate along non-continuous radial fibers generated from the oRG [22]. The expanded area of the oSVZ is considered a new niche of progenitors and is defined by the expression of specific extracellular matrix proteins, that dictate the physical properties of the environment and can be involved in the formation of folds in the human brain [20, 30]. This proliferative niche is also signified by the activity of multiple signaling pathways, YAP-Hippo, Notch, and

mTOR [22, 24, 31], and is exposed to diffusible signals from the cerebral spinal fluid (CSF) [32]. Whereas the Notch pathway is highly expressed in the human ventricular zone and the outer subventricular zone, genes regulating the mTOR signaling pathway are enriched expression in oRG. The enrichment of the mTOR pathway in the outer radial glia appears to be a human-specific feature as it is not observed in developing non-human primate cortex [33]. YAP expression and activity are high in ferret and human oRG, but are low in mouse basal progenitors [31].

Most of the inhibitory or GABAergic neurons are born in the embryonic subpallium, the ganglionic eminences, and to reach their final destination in the cerebral cortex they migrate in a tangential fashion along several stereotypic routes (Figure 1B) [34]. These interneurons are diverse and are usually subdivided to classes based on their morphology and on the expression of several calcium binding proteins. There is an interesting coordination between birth of the interneurons and the excitatory neurons. In mouse models it has been demonstrated that protein deglutamylation controls the pausing of migrating cortical interneurons, and interference in this process affects not only the size of the migrating cohort, but also the generation of age matched interneurons [35]. In humans, there is an expanded oSVZ also in the ganglionic eminences, and in particular the caudal ganglionic eminence is increased in size. These features allow for the higher proportion of interneurons that populate the human brains [24, 36]. Another human-specific phenomenon is the continuation of an extensive migration of interneurons during the early postnatal period, which has not been observed in rodents [37].

2. Recent 3D modeling of human neuronal migration diseases using human brain organoids

LIS1 (*Lissencephaly 1*) has been the first gene to be identified and associated with a neuronal migration disorder [38, 39]. Patients with deletions within one allele of the *LIS1* gene will exhibit lissencephaly, a relatively smooth brain accompanied with simplification in

the layered structure of the cerebral cortex, as well as abnormal positioning of both excitatory and inhibitory neurons within the formed layers. Patients with larger genomic deletions exhibit a more severe phenotype and are defined as "Miller-Dieker syndrome" (MDS) patients [40, 41]. Two studies have used inducted pluripotent stem cells (iPSCs) from MDS patients to generate brain organoids [42, 43].

In the study by Iefremova *et al*., iPSCs were derived from two patients with hemizygote deletions in *LIS1* and *YWHAE.* Rescue lines expressing either *LIS1* or *YWHAE* in the safe harbor *AAV1* locus that allows stable, long-term transgene expression were used as controls [42]. Forebrain organoids from the MDS patients were smaller, showing premature differentiation, a reduced acetylated tubulin network, and a premature transition from symmetric to asymmetric division of the apical radial glia. The cellular organization was partially recovered in the rescue line derived organoids. Interestingly, the authors detected changes in N-Cadherin/ß-Catenin signaling, and activation of the pathway in MDS organoids partially rescued the phenotype.

The study by Bershteyn *et al*. used iPSCs derived from three MDS patients to generate 3D cerebral organoids (Figure 2A) [43]. As in the previous study, the MDS organoids were smaller and exhibited increased apoptosis. The apical surface of the RG was less organized and the angle of live neuronal stem cell divisions was changed, favoring horizontal cleavage plains. To further investigate possible migration deficits, the organoids were plated on Matrigel, which induced extension of processes out of the organoids, and neurons migrated along these processes. Less MDS migrating neurons were noted and their average speed was markedly reduced. A complete rescue of the large deletion was obtained by a duplication of chromosome 17 and organoids from these rescued cells did not differ from the wild-type ones. In addition, the authors placed fluorescently labeled iPSC derived neurons on cortical tissue explants, whereas the wildtype neurons reached the pial surface of the explant, only

10% of the MDS ones accomplished this task, thus demonstrating a cell autonomous component of this disease. The mitotic behavior of the MDS oRG translocated to a further distance during mitotic somal translocation, but remained in mitosis for longer periods of time. This particular behavior was previously noted when primary oRG cells were treated with a microtubule-depolymerization agent [44].

A study by Karzbrun et al. used *LIS1* heterozygous ES cells that were genome edited using CRISPR/Cas9 (Figure 2B) [45]. Using a fabricated device that limited the growth of the brain organoid in the z-axis they could observe the formation of folds in the neuroepithelium [46]. Differential growth may provide a partial explanation for this phenomenon. This phenomenon has been observed in mathematical models, computer simulations, and swelling gels models [46, 47]. Whereas the *LIS1* mutant organoids were similar in size to the wildtype ones, they exhibited overall less but larger folds, mimicking the lissencephalic brain. Measurements conducted on both embryonic stem cells and neuronal stem cells indicated that the *LIS1* mutant cells exhibit a reduced elastic modulus that may in part explain the relative smooth appearance. RNA-seq experiments indicated that the main gene module that changed in the mutant organoids were related to the building blocks of the extracellular matrix and remodeling of the extracellular matrix. The unique device together with the addition of genetic fluorescent markers for nuclei and the cytoskeleton enabled long-term live imaging. The time-lapse images of interkinetic nuclear motility revealed reduced velocity in the apical direction in the *LIS1* mutant organoids. This observation could be explained by the known LIS1 effect on the molecular motor cytoplasmic dynein [38]. In addition, whereas the wildtype nuclei were largest and spent most of the time in the periphery of the organoid during S-phase, the mutant organoids did not obey this rule.

Periventricular Heterotopia (PH) is another brain malformation in which neurons fail to migrate and are retained close to their birth location. Klaus et al. studied the effects of

mutations in the cadherin receptor–ligand pair *DCHS1* and *FAT4* using either brain organoids derived from patients' iPSCs or wild-type and isogenic knockout iPSC lines (Figure 2C) [48]. Previous mouse models exhibited overproliferation of progenitors, quite different from the patients' phenotype [49]. The germinal zones of *FAT4*-mutant organoids were poorly organized. Neurons were observed within the proliferation zones, their processes were affected and displayed a twisted morphology [48]. The mutant organoid displayed neuronal nodules mimicking the main PH phenotype. *DCHS1*-mutant organoids were somewhat better organized, but neuronal processes were abnormal. Sc-RNA-seq showed that the *DCHS1* and *FAT4* transcriptomic profiles were similar and differed from the wild type. Mutant progenitors have undergone premature differentiation, which also fits with the observed phenotype. To specifically examine neuronal migration, organoids were electroporated with microRNA targeting either *DCHS1* or *FAT4* and a week afterwards organoid slices were subjected to time-lapse imaging and were analyzed for three parameters: velocity, number of resting time points and tortuosity. The derived data was then clustered according to distinct cell migration behaviors. Whereas the control neurons exhibited two types of migration behaviors and clustered in two groups, the mutant ones exhibited an additional third type of migration behavior and a third cluster was formed on top of the two control clusters. This finding may assist in explaining how only a fraction of the mutated neurons fail to migrate. Sc-RNA-seq revealed that some of the mutated neurons exhibited an altered RNA expression profile. More specifically, they upregulated genes such as *ROBO3* or *CNTN2* that are involved in processes like axon guidance, neuronal migration and patterning, and downregulated genes that are involved in synapse formation, ion channel, axon guidance and cytoskeleton, some of which are associated with epilepsy, another common feature present in the patients.

Most of the modeling of neuronal migration diseases in brain organoids has been largely restricted to the forebrain, thus missing the contribution of the GABAergic inhibitory neurons that migrate from the ventral to the dorsal forebrain. To enable the integration of GABAergic neurons in forebrain organoids, several groups introduced the concept of "fusion organoids", where two organoids or spheroids are initially cultured individually to produce either forebrain or ventral brain identity, and then once they are placed together, they fuse to form a single organoid. Within this fused organoid, the interneurons migrate tangentially to integrate in the forebrain part [50-52]. The migration was dependent on CXCR4 activity [50], and these organoids exhibited neuronal activity [51, 52]. The study by Birey *et al*. [51] used this setup to investigate interneuron migration deficits in organoids derived from Timothy syndrome patients with mutations in the L-type calcium channel gene *CACNA1C* (Figure 2D). Timothy syndrome is a brain neurodevelopmental disorder associated with autism spectrum disorder and epilepsy [53]. The role of L-type calcium channels in interneuron migration has been previously demonstrated in the mouse brain [54]. Calcium imaging of Timothy syndrome -derived neurons showed increased residual calcium following depolarization. In the fusion organoids, Timothy syndrome interneurons exhibited a less efficient migration pattern with increased saltation frequency. Interestingly, the migration defect was rescued using therapeutic agents targeted to L-type calcium channels.

3. Conclusions & Future Prospects

The modeling of human neuronal migration diseases in brain organoids is at an early stage. It will be interesting to examine additional neuronal migration processes, such as the wide-spread migration of CR cells and migration of neurons to the olfactory bulb. With the development of organoid models and sophisticated live imaging techniques it is now possible to explore these migration events and dissect out the molecular mechanisms underlying this process. So far studies enabled tracking of the mitotic behavior of both RG and oRG

progenitors as well as parameters of interkinetic nuclear motility and somal translocation. In addition, the migration of pyramidal neurons as well as interneurons were studied, yet at this time at a rudimentary stage. Advances in live-imaging which will enable non-invasive image acquisition will benefit this line of studies. Additional integration of activatable live cell reporters in the system will enable to follow for example neurons that are born at a specific time point, or to enable live monitoring of signaling pathways that participate in neuronal migration regulation.

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Annotated References:

* Riva, M., et al., *Activity-dependent death of transient Cajal-Retzius neurons is required for functional cortical wiring.* Elife, 2019. **8**.

The study focused on the mechanisms involved in the elimination of the three distinct Cajal-Retzius linages and further studied the consequences of escaping elimination on cortical circuitry.

** Fujita, I., et al., *Endfoot regeneration restricts radial glial state and prevents translocation into the outer subventricular zone in early mammalian brain development.* Nat Cell Biol, 2020. **22**(1): p. 26-37.

The study focuses on how the process of the regeneration of the apical end feet of radial glia following division is critical in determining the fate of the daughter cells. The ability to regenerate the apical end feet diminishes as development persists and coincide with the formation of oRGs.

** Ohtaka-Maruyama, C., et al., *Synaptic transmission from subplate neurons controls radial migration of neocortical neurons.* Science, 2018. **360**(6386): p. 313-317.

The study found that the initial multipolar-to-bipolar transition occurs following an intimate encounter of the migration neurons with the population of preexisting subplate neurons.

* Kostic, M., et al., *YAP Activity Is Necessary and Sufficient for Basal Progenitor Abundance and Proliferation in the Developing Neocortex.* Cell Rep, 2019. **27**(4): p. 1103-1118 e6.

The study suggests that increased YAP activity in the basal progenitors is sufficient to induce features that are hallmarks of an expanded neocortex.

* Silva, C.G., et al., *Cell-Intrinsic Control of Interneuron Migration Drives Cortical Morphogenesis.* Cell, 2018. **172**(5): p. 1063-1078 e19.

The manuscript describes that a post-translational modification of myosin light chain (MLC) kinase contributes to regulation of migration of the inhibitory neurons.

** Klaus, J., et al., *Altered neuronal migratory trajectories in human cerebral organoids derived from individuals with neuronal heterotopia.* Nat Med, 2019. **25**(4): p. 561-568.

The manuscript describes the use of cerebral organoids derived from iPSCs that enabled modeling of periventricular heterotopia. They showed defects in neuronal migration and a shift in the transcriptome signature of vRG-like cells from patient-derived organoids to an oSVZ/iSVZ-like identity.

** Karzbrun, E., et al., *Human Brain Organoids on a Chip Reveal the Physics of Folding.* Nat Phys, 2018. **14**(5): p. 515-522.

The study describes a device in which the growth of the organoids is limited in the Z axis, this enabled long term imaging and better survival. The organoids grown in this device developed folds which could be used a a model for brain folds and was used to model the smooth brain, lissencephalic phenotype, observed in patients with mutations in *LIS1*.

Figures

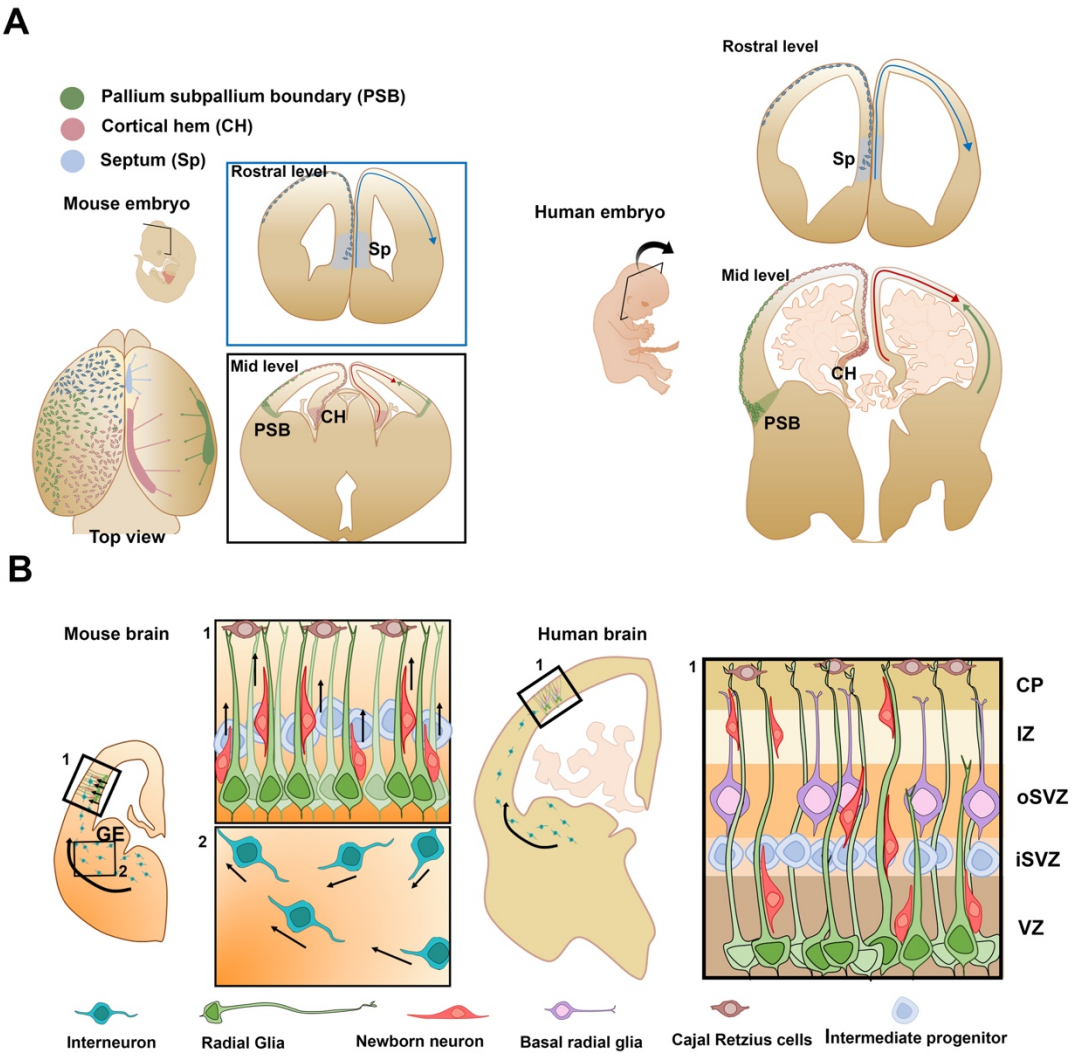

Figure 1: Schematic presentation of migratory routes in the developing mouse and human brain. (A) In mouse and human fetal brain there are at least three sites of origin of the Cajal Retzius (CR) cells; namely 1) the cortical hem (CH), 2) the septum (SP) and 3) the pallial sub pallial boundary (PSB). CR cells derived from all these three regions differ in gene expression, morphology and the undertaken migratory route. Despite certain similarities the kinetics and timeline of migration differ between human and rodents. (B) Both in human and rodent brains new-born neurons exhibit radial migration along the scaffold of RG processes

and the interneurons display tangential migration from the ganglionic eminences (GE) to the cortical plate.

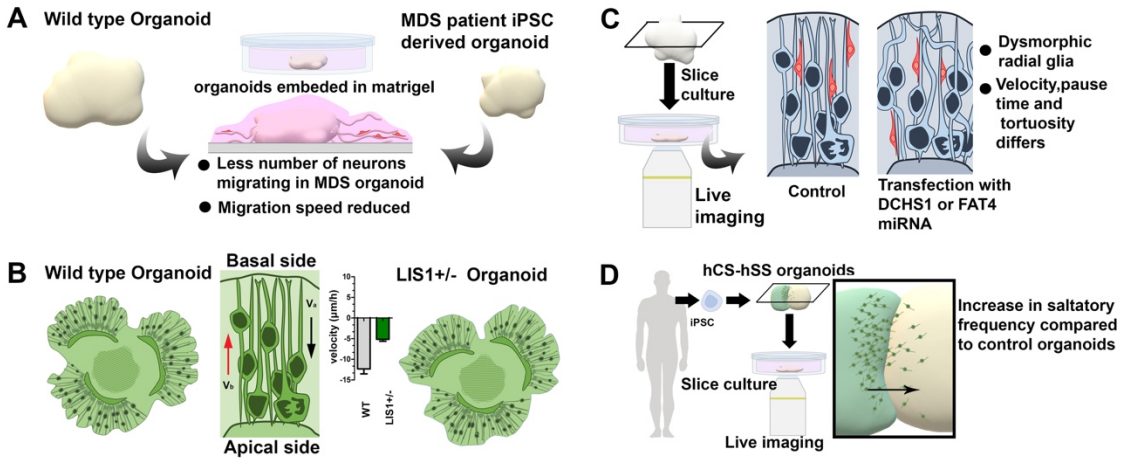

Figure 2: Use of organoid models to understand migration defects underlying neurodevelopmental disorders. (A) Miller-Dieker syndrome (MDS) patient's iPSC-derived organoids are smaller in size compared to control and the organoid derived neurons exhibit aberrant migration when placed in Matrigel [42, 43]. (B) The *LIS1* mutant organoids recapitulate features of lissencephaly where RG progenitors of the mutant organoid display defects in interkinetic nuclear movement [45]. (C) In organoid based models of the neurodevelopmental disease Periventricular heterotopia, the RG appear dysmorphic and various aspects of neuronal migration including velocity, pause time and tortuosity are altered [48]. (D) In the patient derived fusion organoids of Timothy syndrome migration defect of the interneurons was observed.